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APOL1 and Kidney Disease: From Genetics to Biology

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Abstract

Genetic variants in the *APOL1* gene, found only in individuals of recent African ancestry, greatly increase risk of multiple types of kidney disease. These *APOL1* kidney risk alleles are a rare example of genetic variants that are common but also have a powerful effect on disease susceptibility. These alleles rose to high frequency in sub-Saharan Africa because they conferred protection against pathogenic trypanosomes that cause African sleeping sickness. We consider the genetic evidence supporting the association between *APOL1* and kidney disease across the range of clinical phenotypes in the *APOL1* nephropathy spectrum. We then explore the origins of the *APOL1* risk variants and evolutionary struggle between humans and trypanosomes at both the molecular and population genetic level. Finally, we survey the rapidly growing literature investigating APOL1 biology as elucidated from experiments in cell-based systems, cell-free systems, mouse and lower organism models of disease, and through illuminating natural experiments in humans.

INTRODUCTION

Kidney Disease in African Americans

In the United States, 660,000 people have end-stage kidney disease (ESKD), requiring some form of kidney replacement therapy to sustain life (dialysis or kidney transplantation) (1). African Americans develop kidney failure at fourfold-higher rates than European Americans (1, 2). Although only about 12% of the US population is African American, this group comprises just under 40% of the ESKD patients. There are few, if any, other common diseases that show such a marked racial disparity. Although less well documented outside of the United States, the increased susceptibility to kidney disease seems to reflect recent African ancestry and is not specific to Americans (3–5).

The disparity in rates of kidney disease is observed for a variety of subtypes of ESKD and chronic kidney disease (CKD). This includes what is often called hypertension-associated or hypertension-attributed ESKD (H-ESKD), the histologically defined entity focal segmental glomerulosclerosis (FSGS), and pathogen-triggered HIV-associated nephropathy (HIVAN). Despite considerable research into the causes of high rates of ESKD in African Americans over many decades, it was not expected that all of these disease entities would share the same exceptionally strong genetic risk factor or that the discovery of this genetic factor would also change how we think about the relationship between these quite different forms of kidney disease.

Identification of a Major Risk Locus on Chromosome 22

In 2008, two studies took advantage of this racial disparity in the prevalence of kidney disease to identify a locus on chromosome 22 that conferred most or all of the increased risk for nondiabetic kidney disease in African Americans (6, 7). Admixture mapping (also known as MALD, for mapping by admixture linkage disequilibrium) uses differences in genomic sequence between populations with different susceptibility to specific diseases to determine chromosomal regions that are likely to harbor disease-causing genes. These studies observed an excess of western African ancestry among African Americans with FSGS or H-ESKD at this single locus on chromosome 22, demonstrating a genetic basis for the observed ancestry-related disparity in kidney disease prevalence. Importantly, no other such independent loci were identified either in these or subsequent studies. The genomic interval containing the powerful genetic risk factor on chromosome 22 spanned many genes, and the precise variant or variants conferring increased risk of kidney disease were not immediately apparent.

Genome-Wide Association Studies Identify the *APOL1* Risk Variants

Two advances helped lead to the identification of convincing causal variants. First, new genomic resources such as the 1000 Genomes Project established a catalog of human variation from diverse populations that allowed for association testing of African-only or predominantly African variants. Second, tools to analyze the effect of natural selection on the genome were becoming increasingly useful as genome-wide variation data from more populations became available. Analyses to detect signatures of natural selection in African populations suggested a powerful selective sweep had occurred at the chromosome 22 locus associated with kidney disease. The knowledge that natural selective pressures were acting differently on the disease-associated and nondisease-associated haplotypes expanded the size of the genomic locus on chromosome 22 where the risk variants were most likely to be located. In 2010, two distinct coding variants in the *APOL1* gene on chromosome 22 were discovered that appeared likely to explain the association of kidney disease with this genomic locus (8, 9). The basic findings of these studies have been widely replicated (10). Multiple

population-based studies have subsequently demonstrated that these *APOL1* variants account for much of the excess risk of nondiabetic kidney disease in African Americans compared to European Americans (11, 12). Studies in Africans (as opposed to African Americans) show that this is not a geographically limited association (4).

Both of these two genetic variants lead to amino acid changes that alter APOL1 function. The first, G1, codes for two amino acid substitutions near the C terminus that nearly always occur together, S342G and I384M [dbSNP (Single Nucleotide Polymorphism Database) numbers rs73885319 and rs60910145]. The second, G2 (rs71785313), is a six-base-pair deletion leading to loss of amino acid residues 388N and 389Y. Inheritance of two risk alleles (one from each parent) leads to a markedly increased risk of kidney disease, whereas inheritance of one risk allele causes only a very small increase in risk. *APOL1* variants increase the risk of multiple subtypes of kidney disease. Case-control odds ratios are approximately 7 to 10 in H-ESKD (end-stage kidney disease), 10–17 in FSGS, and 29 to 89 for HIVAN (8, 13, 14). With allele frequencies in African Americans of 23% and 15%, G1 and G2 are among the most powerful common risk variants yet identified. Most people now refer to the most common nonrisk allele (wild-type) as G0 and the two risk variant forms as G1 and G2.

The two SNPs that together constitute G1 are in almost perfect, but not 100%, linkage disequilibrium. About 1% of haplotypes with the G342 allele do not have the M384 allele (15). The G342 allele, in the absence of M384, is thought to have the same effect on disease risk as the two variants together (13, 16). G0 is best thought of as the group of haplotypes that does not contain G1 or G2 (17). Other noncoding and coding variants can be used to subdivide G0 into additional haplotypes (16). These haplotypes have not been shown to influence kidney disease susceptibility. In particular, one specific haplotype sometimes referred to as G3 contains nonsynonymous amino acid changes that do not alter susceptibility to kidney disease in African Americans (18, 19).

APOL1-Associated Kidney Disease Follows a Recessive Pattern of Inheritance

Inheritance of risk of kidney disease conferred by the G1 and G2 *APOL1* variants follows a largely recessive pattern. A much smaller effect is often observed in G1 heterozygotes that has not been seen in G2 heterozygotes (8, 13, 14). Inheriting one copy of the G1 allele lowers the age of hemodialysis initiation in nondiabetic ESKD to an age that is intermediate between people with 0 and those with 2 risk alleles (20, 21). In the case of HIVAN, the odds ratio of disease in the setting of HIV infection has been reported to be as high as 5 in heterozygotes (driven entirely by G0/G1 heterozygotes) and 89 in homozygotes (14). Despite these exceptions, most studies have confirmed the recessive nature of kidney disease risk inheritance. Typically, recessive inheritance is associated by a loss-of-function effect on the encoded protein, as discussed below. However, small differences between G1 and G2 associations and phenotypes are an argument against a complete loss of function effect of these alleles (4, 20, 22) (**Table 1**).

APOL1-Associated Kidney Phenotypes

The *APOL1* risk genotypes were originally identified in African Americans with FSGS and/or H-ESKD (8, 9). In contrast to H-ESKD, FSGS is a histopathologically defined form of kidney injury. The accompanying clinical phenotype is typically characterized by abnormally high protein loss in the urine, the accumulation of interstitial fluid in the body (edema), and declining kidney function. FSGS is usually thought to arise from dysfunction of the podocyte, a highly differentiated epithelial cell that forms the distal component of the glomerular filter. This association between *APOL1* genotype and FSGS exists across the age spectrum (23).

Table 1 Difference observed between the G1 and G2 *APOL1* kidney risk variants

Observation	G1	G2	Reference(s)
Binding to SRA	Modestly reduced	Markedly reduced	8, 16
Trypanosoma brucei rhodesiense susceptibility	Not observed in humans to date	Strong protection	40, 41
Trypanosoma brucei gambiense susceptibility	Decreased susceptibility to disease (promotes asymptomatic carriage)	Increased susceptibility to disease	40, 41
H-ESKD risk	Small increase in heterozygote risk	No observed increase in heterozygote risk	8
FSGS/HIVAN risk	Potentially substantial increase in heterozygote risk (OR 2–5)	No observed increase in heterozygote risk	13, 14
Age of ESKD	Heterozygotes develop ESKD at younger age than G0/G0	Heterozygotes do not develop ESKD at a younger age than G0/G0	20, 21
Interferon-associated *APOL1* nephropathy	Present in many individuals with this disease entity	Present in all documented individuals with this entity to date; allele frequency greater than for other types of FSGS relative to G1	22

Abbreviations: ESKD, end-stage kidney disease; FSGS, focal segmental glomerulosclerosis; H-ESKD, hypertension-associated ESKD; HIVAN, HIV-associated nephropathy; OR, odds ratio; SRA, serum resistance antigen.

Other types of kidney disease with differing degrees of relatedness to FSGS share *APOL1* as a powerful risk factor. Hypertension-attributed ESKD, historically considered a chronic vascular disease, is strongly associated with *APOL1* risk variants (24). By far the strongest association of *APOL1* high-risk genotype is with HIV nephropathy, an infectious etiology of kidney disease that includes some histopathologic similarities in common with the most aggressive form of FSGS, collapsing nephropathy (13, 14). Subsequently, severe forms of lupus-associated kidney disease (25, 26) and subtypes of membranous nephropathy (27) have been shown to be associated with the high-risk *APOL1* genotype, especially when there are concomitant features of collapsing nephropathy at the histological level. The observations that the same genetic variants lead to increased risk of types of kidney disease generally thought of as different entities suggests similarities, or perhaps substantial overlap, in the molecular mechanisms of disease pathogenesis. These diseases may be best thought of on a spectrum of *APOL1* nephropathy. The relationship between *APOL1* genotype and diabetic nephropathy (DN) remains incompletely defined. The *APOL1* risk genotype appears to associate with progression of kidney dysfunction in established DN but not incidence of this condition (11, 28). Because most patients with DN do not receive their diagnosis by means of kidney biopsy, and because both *APOL1* kidney disease and DN are quite common, there are undoubtedly many African American individuals included in DN studies who may have *APOL1*-associated kidney disease that is misattributed to DN. Preeclampsia, a disorder of pregnancy characterized by proteinuria, hypertension, and glomerular endotheliosis, is seen at increased frequency in blacks. Recently, an increased risk of preeclampsia in pregnant women was found to be associated with a high-risk *APOL1* genotype in the fetus, but not the mother, providing at least a partial explanation for this racial disparity (29).

These findings remind us that histologic patterns of injury and disease etiologies are different ways of looking at kidney dysfunction. As an example, a large fraction of ESKD in African

Americans has been traditionally attributed to the effects of hypertension. The overlap in the major molecular driver of H-ESKD with FSGS, typically characterized as a disorder of podocyte function, raises the possibility that in individuals with a high risk *APOL1* genotype, hypertension is a symptom of primary microvascular disease rather than the cause. This may explain the relatively limited effectiveness of blood pressure control in slowing the progression of hypertension-attributable kidney disease, especially among those with a high-risk *APOL1* genotype (28). The *APOL1* genotype is a more important predictor of decline in kidney function in CKD attributed to hypertension than is the intensity of blood pressure control. Thus, two variants in one gene, *APOL1*, can contribute to kidney phenotypes that are aggressive or indolent, proteinuric or non-proteinuric, and inflammatory or noninflammatory.

APOL1 and Kidney Transplantation

APOL1 genotype influences the clinical course in kidney transplant recipients. The *APOL1* genotype of a deceased-donor kidney impacts the fate of the transplanted kidney, as kidneys with high-risk *APOL1* genotype tend to fail earlier than do kidneys with 0 or 1 high-risk *APOL1* alleles (30). By contrast, a high-risk *APOL1* genotype in a kidney transplant recipient does not appear to alter the survival of the transplanted kidney (31). These findings suggest that kidney-expressed APOL1 rather than APOL1 circulating in the serum is the major driver of *APOL1* kidney disease. The high-risk *APOL1* genotype is also associated with a higher rate of kidney function decline in kidney transplant donors than low-risk genotypes (32). How *APOL1* genotyping should be utilized in the evaluation of kidney transplant donors is the subject of great interest and, at present, lack of consensus (33, 34). A new US National Institutes of Health (NIH)-sponsored trial will follow transplant and transplant donor outcomes as a function of *APOL1* genotype in an attempt to answer many of the outstanding questions regarding this topic (35).

POPULATION GENETICS OF *APOL1*

There are few examples in complex disease genetics of variants that are common and also have large effects on disease susceptibility. About 13% of African Americans have the *APOL1* high-risk genotype (two risk alleles) and these individuals have a 3- to 30-fold increased risk of various forms of kidney disease. The high frequency of a deleterious genetic variant is surprising. Understanding this unusual combination of allele frequency and effect size requires looking back to the origins, function, and history of these genetic variants in sub-Saharan Africa over the past several thousand years.

APOL1: The Trypanolytic Factor of Human Serum

Humans have innate resistance to some members of the *Trypanosoma* genus of parasites. For example, *Trypanosoma brucei* causes sickness and death in cattle and some herd animals in sub-Saharan Africa, whereas humans are immune. Immunity is conferred by two circulating protein complexes called trypanosome lytic factors, TLF1 and TLF2 (36). A key component of these complexes is the APOL1 protein, which is taken up by trypanosomes scavenging for heme and lipids, whereupon APOL1 lyses the trypanosome based on mechanisms discussed further below. The essential nature of APOL1 in protection from *Trypanosoma* was highlighted when an otherwise healthy farmer from India developed an infection with a normally benign trypanosome (*Trypanosoma evansi*) and was found to have inactivating mutations in both of his *APOL1* alleles and no detectable circulating APOL1 protein (37).

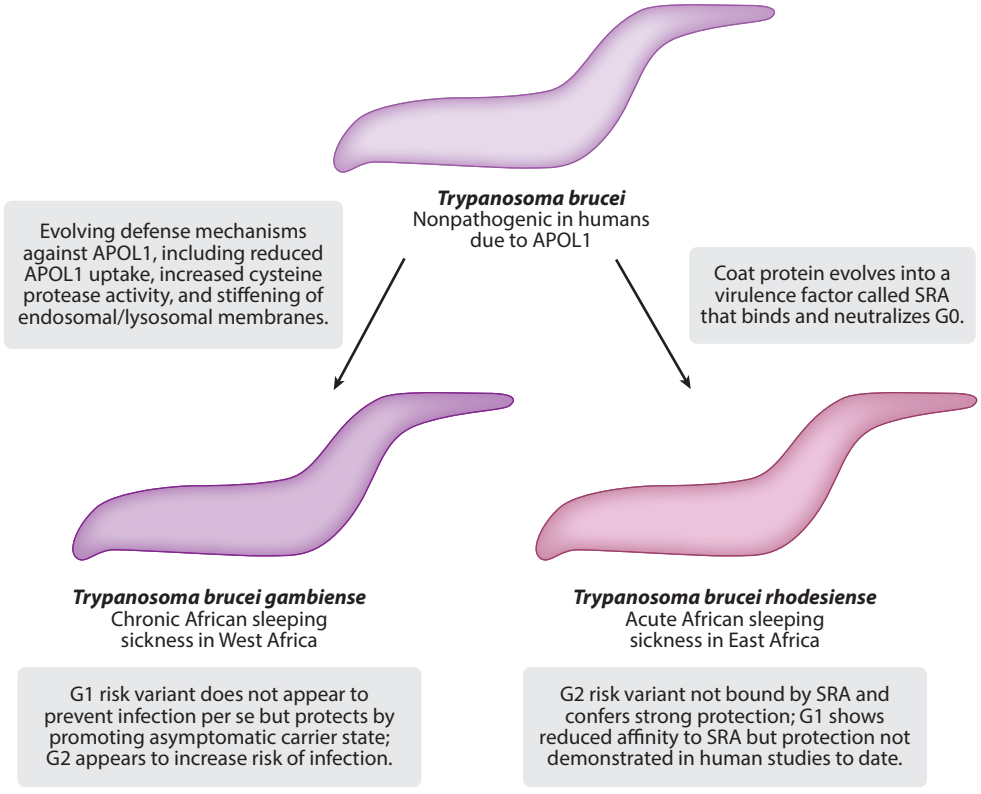

Figure 1

The evolutionary struggle between *Trypanosoma* species and humans. *Trypanosoma brucei* (*T. br.*) does not infect humans because it is susceptible to lysis by circulating APOL1 that it takes up from human plasma. *T. br. rhodesiense* emerged as a human pathogen when it evolved a virulence factor called serum resistance antigen (SRA) that binds to and inactivates APOL1. In in vitro studies, G1 APOL1 has reduced affinity for SRA binding, while G2 shows almost complete absence of binding to SRA. Population studies in humans have demonstrated a strong protective effect of G2 but not for G1 against *T. br. rhodesiense*. *T. br. gambiense* evolved three complementary mechanisms that contribute to its resistance to APOL1/TLF: (*a*) mutations in the trypanosomal receptor for TLF, (*b*) emergence of a *T. br. gambiense* specific glycoprotein (TspGP) that may stiffen the trypanosomal endosomal/lysosomal membranes and prevent APOL1-mediated perforation, and (*c*) a not yet fully characterized factor with cysteine protease activity. The G1 variant does not prevent infection (as defined by seropositivity) to *T. br. gambiense* but does reduce symptomatic African sleeping sickness infection. In contrast, G2 increases the risk of severe disease from *T. br. gambiense*. While recent data have helped explain why the G1 variant is very common in West Africa, other mysteries remain, such as why G2 is still as or more common in West Africa than in East Africa. A deeper understanding of environmental conditions over time, changing tsetse fly (the brucei trypanosome vector) distribution, historical migrations of African peoples, the effects of sleeping sickness eradication campaigns, and protection conferred by APOL1 against other pathogens would permit a clearer window into this complex evolving story.

At some point in the last 10,000 years, two trypanosome subspecies evolved in Africa from *T. brucei* that were pathogenic to humans (**Figure 1**). *T. brucei* (*T. br.*) *rhodesiense* is found east of the Rift Valley and causes acute African sleeping sickness. In *T. br. rhodesiense*, a coat protein was repurposed into a virulence factor called SRA (serum resistance antigen) that can bind and inactivate APOL1 protein inside the trypanosome, overcoming the protection against trypanosomes normally conferred by APOL1 (38). *T. br. gambiense* is another pathogenic subspecies found

predominantly west of the Rift Valley that causes chronic African sleeping sickness. The *T. br. gambiense* subspecies evolved a more complex suite of defenses against APOL1, including several diverse mechanisms of action (39). The existence of multiple mechanisms of resistance to APOL1 in *T. br. gambiense* may have evolved because humans are thought to be the sole, or at least the predominant, reservoir for this subspecies.

The high frequency of the deleterious *APOL1* kidney risk alleles strongly suggests that they also have beneficial properties. Two different approaches to evaluating genetic variants for positive selection, or an increase in frequency due to advantageous properties, support this viewpoint. First, in populations with a high frequency of the risk alleles, risk variants G1 and G2 are on larger genomic haplotypes than nonrisk *APOL1*, with longer expanses of a uniform, nonrecombined flanking DNA sequence surrounding either G1 or G2 than that surrounding the wild-type G0 alleles. As the DNA sequence near the mutation acts as a molecular clock that marks time via recombination events, this finding indicates that risk alleles rose in frequency very quickly within the last 10,000 years. Second, the G1 variant in particular shows exceptionally strong differentiation in frequency between populations in Africa (much higher in West Africa than East Africa) that are otherwise very similar genetically. The rapid emergence of this geographic differentiation again points toward some evolutionary force favoring an increase of risk variant frequency in some environmental conditions.

Initial functional studies in vitro and in vivo demonstrated that both G1 and G2 variants were more effective at killing pathogenic *T. br. rhodesiense* (but not *T. br. gambiense*) than G0 variants, with G2 generally more potent than G1 at trypanolysis. Though supporting a history of a selective sweep by the risk variants, these findings did not answer some key questions. Specifically, the combined frequency of the risk variants is much higher in West Africa, whereas the pathogen they purportedly protect against is, at least currently, limited to East Africa.

Some provocative findings in human studies from Africa have helped build a more coherent model of the history of the *APOL1* risk alleles. Investigators found a strong protective effect of G2 against *T. br. rhodesiense* infection in case-control studies, consistent with trypanolysis studies in culture and in mice; no effect for G1 was observed (40, 41). However, the effects of *APOL1* risk variants against *T. br. gambiense* were unexpected but illuminating. G2 appeared to predispose to symptomatic *T. br. gambiense* infection, whereas G1 did not prevent infection per se but did appear to protect against symptomatic infection (i.e., it led to asymptomatic carriage). Taken together, the data support a model where G2 is an older mutation that protects against *T. br. rhodesiense*, whereas G1 is a more recent mutation that arose in West Africa and spread very quickly due to its ability to help humans survive *T. br. gambiense* infection. Additional data are accruing rapidly in African populations that may soon help bring this model into sharper focus.

THE APOL1 GENE AND PROTEIN

APOL1 is a late arrival to a 6-member family of *APOL* genes on chromosome 22 in humans (42, 43). It likely arose from a gene duplication event approximately 30 million years ago. This event occurred after the primate lineage branched off from other mammals. Thus, *APOL1* is not present in the genomes of any mammals besides primates. Furthermore, only some primate genomes encode a functional *APOL1* gene, with a notable absence in our closest relative, the chimpanzee. *APOL1* has not been observed in New World monkeys, leading to the estimated origin 30 million years ago after the split between Old and New World monkeys. APOL1 has been lost or pseudogenized in multiple primate species in the Old World lineage, suggesting that not only is *APOL1* dispensable in certain cases but it may even be detrimental. The absence of *APOL1* from nearly all mammals, and many primates including long-lived species, has important implications

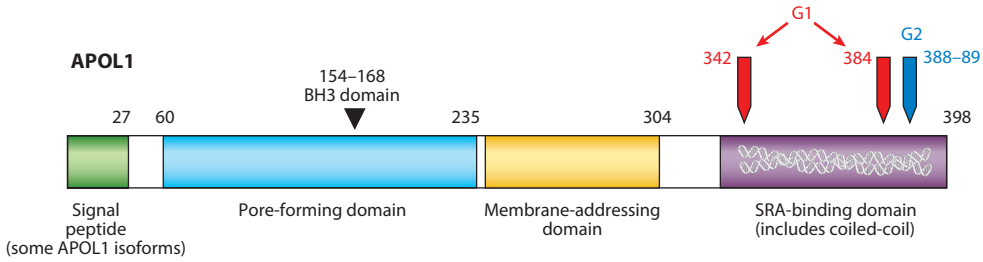

Figure 2

APOL1 protein domains. The most common APOL1 isoform has 398 amino acids and a molecular weight of approximately 42 kDa. This isoform has a signal peptide (*green*) that allows APOL1 export from some cell types. The other domains are named for their role in trypanolysis. The pore-forming domain (*blue*) has structural similarities to bacterial colicins and also contains a BH3-only domain, which suggest a potential role in either autophagy or apoptosis. The membrane-addressing domain (*yellow*) may act as a pH-dependent switch that has been predicted to alter binding to lipids containing particles. The serum resistance antigen (SRA)-binding domain (*purple*) is targeted by the SRA protein of *Trypanosoma brucei rhodesiense*; the G1 and especially G2 human APOL1 variants reduce the affinity of APOL1–SRA binding.

for gain versus loss of function of *APOL1* risk alleles, a topic addressed in the section titled Models of *APOL1*-Associated Disease. Some other species have more *APOL* genes than humans, and it remains possible that some of these other *APOL* genes have functions that overlap with *APOL1*, but exploration of other *APOL* gene function in humans and other species has been minimal.

The characteristic feature of APOL1 that most clearly differentiates it from other APOL proteins is the presence of a signal peptide in some splice isoforms enabling export from the cell (**Figure 2**). Although the most abundant transcripts encode a signal peptide, it is spliced out of some transcripts, leading to intracellularly restricted minor isoforms. The protein itself is organized into three major functional domains defined by their roles in trypanolysis (44). The N-terminal amino acids form the colicin-like domain, named for their homology to bacterial colicins that perforate membranes. Within this domain is a BH3-only subdomain found in BCL2-like proteins that often have roles in apoptosis or autophagy. The central part of APOL1 is called the membrane-addressing domain, a putative pH-dependent apparatus for activating APOL1 in acid environments. The APOL1 kidney risk variants are located near the C terminus in the SRA-binding domain, the target of the *T. br. rhodesiense* SRA virulence factor. The APOL1 kidney risk variants alter SRA binding, with G2 essentially eliminating and G1 reducing this interaction. The SRA-binding domain includes a coiled coil with a leucine zipper motif, creating an amphipathic helix that is important for SRA binding and possibly for interaction with lipids. The effect of APOL1 kidney risk variants on the structure of the C terminus has generated competing structural models with respect to conformational stability (45, 46).

APOL1 circulates at high (micromolar) levels either on HDL3 molecules (TLF1) or in a lipid-poor state complexed to immunoglobulin M (IgM) (36, 38, 47, 48). Other proteins in these complexes include APOA1 and haptoglobin-related protein (HPR). The circulating form of APOL1 is primarily generated by the liver (49). APOL1 is expressed widely in many cell types within many organs including the vasculature, kidney, lung, pancreas, and others (50, 51). APOL1 expression is enhanced in inflammatory settings, consistent with a role as an immune defense molecule. *APOL1* responds to interferons, lipopolysaccharide, Toll-like receptor (TLR) agonists, tumor necrosis factor (TNF), and other cytokines with robust upregulation (22, 52). Its promotor is notable for canonical interferon response elements, a feature it shares with some other *APOL* family genes (22). The *APOL* genes respond in concert to stimuli such as interferon and APOL1 interacts with

other APOL proteins, suggesting complex interdependent behavior (53, 54). Genetic loci associating with circulating APOL1 levels have been identified but no association between circulating APOL1 levels and kidney disease has been demonstrated (55, 56).

APOL1 FUNCTION

Investigators were exploring APOL1 function in trypanolysis long before the discovery of the genetic variants linked to kidney disease. Investigators have reached consensus that APOL1 is an essential part of the trypanolytic machinery, along with other proteins that circulate with APOL1 and appear to potentiate its efficacy (such as HPR), but the molecular mechanisms leading to trypanosomal killing are still debated (57). Initial studies focused on the lysosome, an acidic organelle deemed important for APOL1 activation, proposing formation of ion channels that lead to lysosomal rupture. Investigators put forth evidence for cation versus anion channel activity through a pore formed by, or at least including, APOL1 protein (58, 59). More recently, a competing or perhaps complementary model has suggested that the trypanosome mitochondria may be the target of APOL1-mediated injury (60). Genetic screens have identified several trypanosomal genes (e.g., those encoding KIFC1, V-ATPase) that are necessary for APOL1-mediated trypanolysis, with their roles in transport and acidification holding potential clues to mechanisms underlying kidney disease (60–62).

Cell-free models employing different types of lipid membranes have added to our knowledge of APOL1 function (63). One seminal finding emerged from studies using recombinant APOL1 protein in a lipid bilayer system (64). Investigators demonstrated a compelling two-stage model for APOL1 channel function. First, APOL1 requires activation at acidic pH that initiates a low-conductance cation current. Raising the ambient pH toward more neutral conditions then induces a huge increase in cation conductance. These findings support a model where APOL1 channel function may require activation in an acidic organelle along the endosomal-lysosomal axis, followed by trafficking to nonacidic compartments such as outer membranes or mitochondria where ion channel activity is more potent. A two-step activation process may help limit promiscuous pore-forming activity and injury to the host. Additional work in vesicle-based systems has also proposed the idea of a pH switch, with evidence for both anion and cation flux that depended on both the pH and lipid environment (65).

Initial hypotheses about APOL1 function in mammalian systems, and consideration of the nature of risk-variant dysfunction, followed from both antitrypanosomal biology and human population genetics. The toxic nature of APOL1 in trypanosomes, presumably based on the capacity to create pores in membranes, engendered the idea that APOL1 might do something similar in mammalian cells. In addition, the absence of APOL1 in most mammals makes an essential function for APOL1 in kidney development and homeostasis unlikely, at least under most environmental conditions. The existence of even one APOL1-null human with apparently normal kidney function suggests that this reasoning extends to humans (37).

Experiments in Human Cellular Systems

Many experiments have used overexpression in human cells to test the functional properties of APOL1, using both risk and nonrisk alleles. Results have been perplexing, notable particularly for their inconsistency and sometimes even contradictory nature. This is likely due to the many different cell types studied (HEK293 versus podocytes, transformed versus primary), methods of APOL1 expression (stable transfections versus transient transfections, lipid versus virally delivered vectors, constitutively driven versus tet-regulated expression), variability in APOL1

sequences, and readouts measured. Arguments have been made for enhanced cell death of almost every type by APOL1 risk variants (apoptosis, necrosis, autophagy, pyroptosis, etc.), and counterarguments for no difference in toxicity by genotype at all (66, 67). The mechanisms proposed to initiate APOL1 risk-variant injury are numerous and diverse. Here, we highlight some fundamental questions about the gain-of-function model and some common themes that have emerged in their pursuit.

Does Enhanced Toxicity to Kidney Cells Explain the *APOL1* Risk-Variant Phenotype?

Many studies using a variety of different experimental systems have demonstrated that overexpression of the *APOL1* kidney risk variants causes an increase in cell death (68–75). While some reports also show that overexpression of G0 is associated with some toxicity, it is typically much less than observed with the risk variants. APOL1 has two domains, a pore-forming domain and a BH3-only domain, potentially capable of driving cytotoxicity, but cell death has not been definitively attributed to either of these functional elements. More nuanced phenotypes, such as the effect of APOL1 on the actin cytoskeleton and podocyte differentiation, have also been considered, and differences between *APOL1* genotypes were observed in some cases (76–78). Many assumptions have been made that the podocyte is the main target of APOL1 toxicity, based at least in part on the association between FSGS and podocyte genes in general. These assumptions probably do not capture the full complexity of either the interrelationships between podocytes and other glomerular cell types or the spectrum of *APOL1* kidney diseases that includes presentation with chronic vascular disease phenotypes in addition to heavy glomerular proteinuria.

How and Why Is the Behavior of the Risk Variants Different from G0?

High rates of kidney disease with *APOL1* risk variants versus G0 suggest differences in behavior at a cellular level. Despite many reports of different phenotypes, such as cell death, actin cytoskeletal structure, or mitochondrial dysfunction, the fundamental differences in behavior between the *APOL1* alleles at a molecular level have remained elusive. Rarely have clearly defined experimental differences been replicated.

Understanding G0 versus risk-variant behavior based on intracellular trafficking and localization has relied on overexpression systems that may not permit observation of subtle but important differences. APOL1 staining has been ascribed to many organelles, including endoplasmic reticulum, lysosomes, mitochondria, lipid droplets, and cell membrane (**Table 2**). There are compelling experiments that document adverse APOL1 risk-variant effects on specific organelles, such as mitochondrial dysfunction, but these have not been accompanied by clear differences in APOL1

Table 2 Localization of intracellular APOL1 in cell-based studies

Organelle	Reference(s)
Endoplasmic reticulum	75
Endosomes	68
Lysosomes	68
Autophagosomes	46, 68, 75
Mitochondria	74, 75
Lipid droplets	79
Plasma membrane	59, 72

localization, nor has it been determined where mitochondrial dysfunction fits in the causal chain (i.e., does APOL1 adversely affect the mitochondria directly or is mitochondrial dysfunction downstream of some other APOL1-driven process?) (74, 75, 79).

Similarly, differential binding partners for APOL1 risk and nonrisk variants have yielded no consistent findings to illuminate the mechanism of disease. There has been reasonable speculation that there may be endogenous proteins that bind and restrict APOL1 toxicity in an SRA-like manner or that APOL1 risk variants might traffic via risk variant-specific binding proteins to organelles where toxicity occurs. In cell-free systems, risk and nonrisk APOL1 appear to have similar ion conductances, though unpublished data suggest that differences between genotypes can be evoked in certain experimental systems (64). Differences in ion flux by genotype have been shown in some APOL1-overexpressing cellular systems, but not that APOL1 itself is conducting those currents, whereas similar experimental systems have not observed differences in flux based on genotype at all (66, 72).

Unlike circulating APOL1, where integration into well-defined protein complexes has been confidently documented, there have not yet been intracellular binding partners of APOL1 with clear functional importance. Individual reports for APOL1 interactions that differ by genotype, such as those with αvβ3 integrins and VAMP8, are provocative and await confirmation by independent groups (46, 80). Differential binding between particular *APOL1* genotypes and SRA strongly suggests these differences are likely to exist and might have a major physiologic impact, but their identification has proved challenging. Recent studies of other important proteins associated with genetic disease, such as cystic fibrosis transmembrane conductance regulator (CFTR), where potentially hundreds of binding partners have been observed, highlight the complexity of finding the one or few interactions that drive the kidney phenotypes.

It will likely require more than cell-based models alone to yield comprehensive answers about APOL1 biology and pathology. Looking for intersections between orthogonal systems seems a productive first step in aligning mechanism and relevance to human biology.

What Is the Mechanism of Cell Death and Why Are There So Many Answers?

Many groups have sought to characterize the nature of APOL1-mediated cell death. Early reports from the cancer literature suggested autophagic cell death was important, even in G0 forms of APOL1, which seemed consistent with its BH3-only domain (81). Alterations in autophagic flux have been reported by several investigators, and enhanced apoptosis (another potential BH3-driven process) similarly appears activated in some APOL1-overexpressing cell systems (52, 68, 72, 82). Swelling and necrosis suggestive of pore formation in APOL1-overexpressing cells have also been widely documented (70, 72). Even quite specific cell death mechanisms have been observed, such as caspase-dependent pyroptosis (68). Cell death that can be measured over a few days in culture as a proxy for a much slower process taking place in the full complexity of the human glomerulus has inherent limitations. Even the assumption that cell death is the best experimental readout, as opposed to other phenotypes such as reorganization of the podocyte cytoskeletal elements or alteration of endothelial cell surface proteins, is an open question. These cytotoxicity experiments are likely best viewed as a valuable adjunct to a perspective that incorporates natural experiments in humans, mouse models, and molecular analyses such as protein structure rather than methods that will reveal a coherent explanation of APOL1 dysfunction by themselves.

What Is the Mechanism Behind Recessive Gain of Function?

We consider it a central mystery why *APOL1* kidney risk variants confer risk in a largely recessive manner if they are truly gain-of-function variants. Several explanations have been put forth

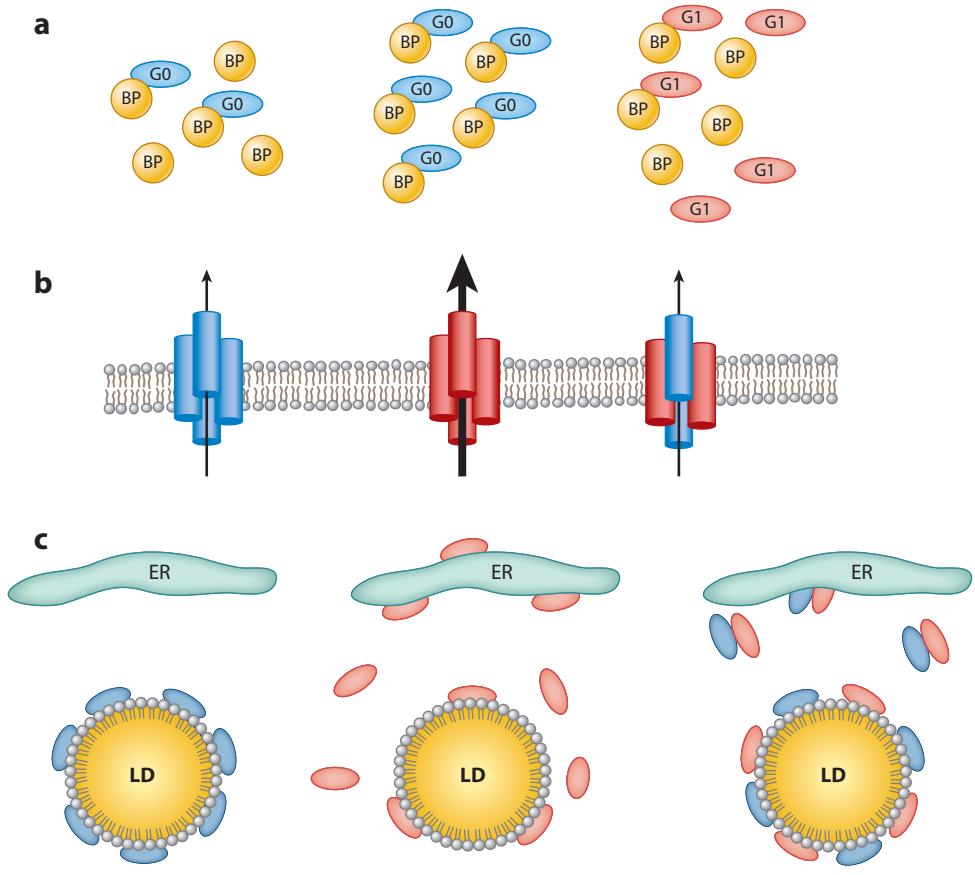

Figure 3

Some potential models explaining recessive loss-of-function. Blue denotes G0, and red shows the risk variant (G1 or G2). (*a*) Threshold effect model. (*Left*) Under normal conditions, physical interaction between APOL1 and a potential APOL1 binding protein (BP) with high affinity for G0 and lower affinity for risk variants can prevent APOL1-mediated toxicity, whether the genotype is G0 or a risk variant, due to excess BP relative to APOL1. In the setting of enhanced APOL1 expression, G0 can still be bound and its toxicity neutralized (*center*), but there is still excess unbound risk variant due to lower affinity, leading to toxicity (*right*). There is possibly some threshold for free risk-variant APOL1 that is not exceeded in heterozygotes but exceeded in homozygotes when APOL1 levels are high. (*b*) Oligomerization model. G0 oligomers, here presented in the form of a multi-subunit channel, may lead to low ion flux. Channels composed of all risk-variant APOL1 generate high flux leading to cell injury. Subunits composed of both G0 and risk variant may behave like G0-only channels. In this model multimerization would still require high APOL1 levels compared to the baseline state. (*c*) G0 rescue model. Lipid droplets (LD) appear to carry more G0 than risk-variant APOL1. G0 efficiently traffics from the endoplasmic reticulum (ER) to the LD. Risk-variant APOL1 is less efficiently trafficked to the LD, leading either to toxicity from excess free APOL1 or to retention of APOL1 in the ER (with ER stress related dysfunction). In the heterozygote state, G0 helps facilitate the trafficking of risk-variant APOL1 from the ER to the LD.

(**Figure 3**). The idea of dose dependence is one obvious possibility. The observation that APOL1 expression can be upregulated tens to hundreds of times with inflammatory factors does pose a challenge to the dose hypothesis because the highly inducible nature of *APOL1* gene expression might overwhelm the twofold difference in gene dosage (16). Others have postulated that APOL1

multimerization might explain recessive inheritance (83). For example, a multi-subunit functional element such as a channel might only confer pathogenic properties if it consisted solely or mostly of risk-variant APOL1. In other words, nonrisk APOL1 could reverse or rescue the toxicity of risk-variant APOL1, probably through direct interaction.

It would not be surprising if two different risk variants such as G1 (where S342G is the critical mutation) and G2 (at amino acids 388–89) in the same general functional domain each caused the same loss-of-function effect. But it is less obvious how they might cause the same gain-of-function (including compound heterozygosity: G1/G2 has the same general phenotype as either homozygous state). Perhaps the most parsimonious explanation is that the SRA-binding region is an autoinhibitory domain, and G1 and G2 cause loss-of-function in the inhibitory domain, leading to overall gain-of-function of the protein. Trypanosomal SRA may have co-opted a preexisting binding domain for some endogenous inhibitory protein(s), and the kidney risk variants are not as avidly bound by either SRA or those endogenous proteins.

MODELS OF *APOL1*-ASSOCIATED DISEASE

Mice

The development of good animal models of *APOL1*-associated disease is complicated by several facts. *APOL1* does not exist in most mammals, including those commonly used to model human disease. Several different strategies have been used to develop mouse models of *APOL1*-associated disease. Bruggeman and colleagues (84) developed a transgenic mouse model in which a nephrin promoter is used to drive podocyte-specific expression of G0 or G2 forms of APOL1. The G2 mice, while lacking an overt kidney phenotype, were found to have a lower podocyte density at age 200 days. The G2 transgenic mice, and to a lesser extent the G0 transgenic mice, displayed a preeclampsia-like phenotype. By contrast, Beckerman et al. (68) developed different transgenic mouse models engineered to display either podocyte-specific or tubular-inducible expression of the G0, G1, or G2 form of APOL1. Podocyte-specific expression of G1 or G2 led to the development of proteinuria and glomerulosclerosis, whereas tubular expression did not. The amount of kidney damage correlated with the level of APOL1 expression. Ryu et al. (85) described the development of BAC transgenic mice, which carry a bacterial artificial chromosome that contains the entire human *APOL1* genomic region. These mice have been used to explore mechanistic questions regarding APOL1 biology. At six months of age, these mice did not demonstrate an overt kidney phenotype.

Techniques other than transgenesis have also been used to study APOL1 and its variants in mice. Several groups have used hydrodynamic delivery of human *APOL1* to mice to study in vivo APOL1 biology (16, 80, 86). Risk-variant APOL1 delivery by this method causes more liver and kidney damage than does the G0 form.

Other Organisms

Other model organisms have also been used to study APOL1 biology and variant effects.

Zebrafish have a single gene that shows homology to the human *APOL* family of genes/proteins, *zAPOL1*. Inactivation of zAPOL1 has been shown to cause defects in the function of the larval pronephric glomerulus in two studies (87, 88). Anderson et al. (87) found that expression of G0 APOL1 in these mutant fish rescued these defects, while G1 did not. Complementation with G2 APOL1 led to developmental defects. In another approach, Olabisi et al. (89) expressed G0 or risk variant APOL1 in a podocyte-specific or endothelium-specific manner.

Transgenic risk-variant fish showed mild ultrastructural changes on electron microscopy absent from G0 fish, but not a more overt phenotype.

Two studies found similar results when APOL1 was overexpressed in the *Drosophila* podocyte-like cell type known as the nephrocyte (90, 91). Nephrocytes function as part of the excretory system and share many structural and functional features with mammalian podocytes. Similar to what has been found in some (but not all) model systems, expression of risk-variant APOL1 in nephrocytes leads to hypertrophy, accelerated cell death, and defective intracellular organelle acidification, in marked excess of what is observed with the G0 form (90, 91). Similar effects were observed in yeast (90).

Xenopus oocytes have been used to study APOL1's function as a membrane protein. APOL1 expression in oocytes was found to increase ion permeability and led to severe toxicity. This toxicity was found to be independent of the BH3 domain in this experimental system (82).

Considering the Loss-of-Function Hypothesis

Though comparative genomics and at least one APOL1-null human argue that APOL1 is not essential for fundamental kidney function, it does not necessarily follow that APOL1 is dispensable for kidney health in some environmental conditions. The best-developed case for loss-of-function has been built around enhanced interactions between G0 with proteins in the endosome/lysosomal system when compared with the risk variants (46). Many other differences in binding preferences between G0 and the APOL1 risk variants are highly likely. Demonstrating loss-of-function when a protein's function is not at all apparent remains a much harder challenge than demonstrating enhanced injury. The possibility that APOL1 kidney risk alleles have properties encompassing both gain- and loss-of-function should not be easily dismissed.

Why the Kidney? Does APOL1 Affect Other Organ Systems?

Why do these APOL1 variants primarily affect the kidney? Although there is conflicting evidence regarding an independent effect of the *APOL1* genotype in the development of hypertension in young people and cardiovascular disease in older people, it is clear that the primary site of *APOL1*-associated disease is the kidney (92–95). APOL1 is widely expressed and circulates at high levels in the bloodstream. Most of the circulating APOL1 is made by the liver (49). What is special about the kidney?

Of course, this question is not unique to *APOL1*. Many widely expressed genes cause largely limited kidney disease when mutated (examples in nephrology include *ACTN4* and *TRPC6*). One explanation may be related to the nature of the glomerular podocyte. Podocytes are terminally differentiated epithelial cells with a very intricate actin cytoskeleton–based architecture. Over 50 genes have been identified, which, when mutated, cause proteinuric kidney disease, many without any accompanying nonrenal features. These include genes important for cell structure, cytoskeletal remodeling, signaling, and mitochondrial function. This suggests that podocytes are sensitive to perturbation from many types of cellular dysfunction that other cells may be resistant to. It is not clear, however, if the site of *APOL1*-induced kidney injury is limited to an effect on the podocyte. In particular, hypertension-associated kidney disease and preeclampsia in association with high-risk *APOL1* genotypes are likely to involve endothelial and/or vascular dysfunction.

APOL1 SECOND HITS: GENES VERSUS ENVIRONMENT

Individuals inheriting the *APOL1* high-risk genotype do not universally develop kidney disease. This observation implies that other factors—second hits—must be required to drive *APOL1*

kidney disease in at-risk individuals. Both genetics and environmental factors, or some combination, are possible contributors.

Genetic studies have tested for modifiers of the *APOL1* risk genotype. In H-ESKD, several candidate gene studies have demonstrated associations of interest (e.g., GSTM, APOL3, hemoglobin/sickle cell trait), though no associations have been identified at genome-wide significance (54, 96, 97). While it is almost certain that other gene variants do modify risk, the studies to date point toward a model of many loci with small effect sizes that will take very large cohorts to identify definitively. In FSGS, where the effect size of *APOL1* risk variants are larger than in H-ESKD and where the phenotype may be more *APOL1* specific, one study found evidence for a modifier locus on chromosome 6 (98). The most compelling causal candidate at this locus was the UBD (*FAT10*) gene, due to the fact that UBD has been associated in other unbiased screens related to *APOL1* phenotypes, including gene expression profiles from risk-variant versus nonrisk human glomerular tissue (55, 99, 100). Experiments support the idea that the ubiquitin-like UBD protein may bind APOL1 and alter its abundance, with African UBD haplotypes associated with lower UBD expression and higher FSGS risk.

Environmental triggers may be even more important. The overwhelming interaction between HIV infection and the *APOL1* risk variants is the most powerful evidence for the primacy of environmental influences, with an odds ratio (OR) of 29–89 and estimates that, in the pre-HAART era, 50% of individuals with the high-risk genotype developed HIVAN (13, 14). Other observations are consistent with a virus or immune response hypothesis. In individuals who developed collapsing glomerulopathy after therapeutic interferon administration in one series, all had a high-risk *APOL1* genotype (22). Given the observation that interferons could upregulate APOL1 expression levels by ten- or hundred-fold in accompanying experiments in cellular systems, one potential model is that both the high-risk *APOL1* genotype and high APOL1 expression (driven in some cases by virus or the innate immune response to virus) may be required for disease to occur. Additionally, associations between viruria and APOL1 kidney disease lend support to the general idea that viruses may activate an APOL1 response capable of initiating kidney injury (101, 102). Reports that APOL1 is a multipurpose viral restriction factor leave open the possibility of loss-of-function biology that requires more investigation (103).

FUTURE DIRECTIONS

Many advances have set the stage for progress in understanding *APOL1* kidney disease. Genetic data from enormous studies seem likely to help identify genetic modifiers, environmental risk factors, and more- versus less-effective therapies, all of which have the potential to teach us about mechanism and inform efforts to prevent or treat disease. New technologies such as CRISPR allow for rapid generation of cell lines and animal models that would have been out of reach only a few years ago. We may even see technologies such as nucleic acid–based therapies or screening of huge DNA encoded compound libraries leading to therapies before we fully understand the mechanism of disease. Thus, there is reason for optimism that these advances in understanding the biology of APOL1 and its genetic variants will ultimately lead to a reduction in the major racial disparities in kidney disease.

DISCLOSURE STATEMENT

M.R.P. and D.J.F. are coinventors on patents related to APOL1 diagnostics and therapeutics. Both individuals receive research support from and have consulted for Vertex Pharmaceuticals and both have equity in Apolo1Bio.

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